BBA 66729

CONVERSION OF THE RED SEMIQUINONE OF D-AMINO ACID OXIDASE TO THE BLUE SEMIQUINONE BY COMPLEX FORMATION

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(Received May 3rd, 1972)

SUMMARY

1. The red semiquinone of D-amino acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) prepared by photoreduction was changed into the blue semiquinone upon anaerobic addition of excess benzoate.

2. Double reciprocal plots between the concentrations of the blue semiquinone formed and those of benzoate gave a straight line, indicating a complex formation between the semiquinoid enzyme and benzoate. The dissociation constant of the complex was $2 \cdot 10^{-2}$ M.

3. Both D- and L-alanine combine with the red semiquinone of the enzyme in competition with benzoate. They both form complexes of the red species.

4. The relation between the dissociation constant of the complex (blue species) formed with *m*- or *p*-substituted benzoate derivative and the empirical value, Hammett's σ (L. P. Hammett, *Physical Organic Chemistry*, McGraw–Hill, New York, 1940, p. 184) gave a straight line in the range of $\sigma = -0.4$–0, indicating the participation of a COO^- group in the formation of the blue species.

5. Anaerobic addition of cinnamate or crotonate to the red semiquinone of the enzyme also gave the blue species.

INTRODUCTION

The observation that the purple complex of D-amino acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) gradually changes into the semiquinoid species upon anaerobic storage in the dark provided an important clue[1,2] in the elucidation of the nature of the purple complex. In an attempt to demonstrate the irreversible conversion of the purple intermediate to the semiquinoid species, anaerobic addition of benzoate to the stored purple complex solution was performed, since it was expected that the substrate moiety of the purple complex would be exchanged with benzoate to yield the oxidized enzyme–benzoate complex. The results indicated that the freshly prepared purple complex was converted to the oxidized enzyme–benzoate complex upon addition of excess benzoate, while the stored one was partly converted, indicating that the semiquinoid species is not involved in the enzyme–

substrate complex[2]. In the latter case, a small increase in the absorbance in the vicinity of 600 nm was noticed. This provoked us to measure the absorption spectrum of the semiquinoid enzyme mixed with excess benzoate. As briefly reported earlier[2,3], the semiquinoid enzyme changed from red to blue upon mere addition of excess benzoate. The blue solution thus obtained represents a similar absorption spectrum to that of 'blue semiquinone' or 'blue radical' of some flavoproteins[4–8], and this blue species of D-amino acid oxidase was considered to be a neutral flavin radical[9]. The present paper deals chiefly with a detailed investigation on the effect of benzoate and its derivatives on the red semiquinone of this enzyme.

MATERIALS AND METHODS

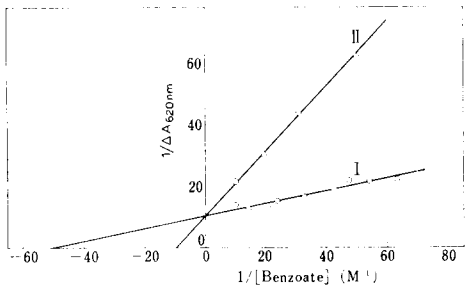
D-Amino acid oxidase was prepared according to Yagi *et al.*[10]. The red semiquinone of the enzyme was prepared in a Thunberg-type cuvette by anaerobic irradiation at pH 8.3 in 0.017 M pyrophosphate buffer containing 0.02 M EDTA according to Massey and Palmer[7]. *o*-, *m*- and *p*-Aminobenzoic acid, *o*-, *m*- and *p*-methylbenzoic acid, *o*-, *m*- and *p*-chlorobenzoic acid, *o*-, *m*- and *p*-nitrobenzoic acid, *p*-methoxybenzoic acid, *p*-hydroxybenzoic acid, *m*- and *p*-iodobenzoic acid, sodium benzoate and D-*α*-amino-*n*-butyric acid were purchased from Sigma Chemical Co., St. Louis. Cinnamic acid, crotonic acid, phenylacetic acid, butyric acid, sorbic acid and DL-*α*-amino-*n*-caproic acid were obtained from Tokyo Kasei Kogyo Co., Tokyo. D-Alanine and L-alanine were purchased from Nakarai Chemicals, Kyoto. All acids were used, without further purification, as their sodium salts.

The enzyme solution was placed in the main chamber of a Thunberg-type cuvette. The reactant (benzoate, its derivative, amino acid or other carboxylate) was placed in the side chamber and taken to dryness by warming the side chamber with suction. Then, the contents of the cuvette were carefully made anaerobic by degassing and flushing repeatedly with argon gas. The gas was previously washed with alkaline pyrogallol solution. The cuvette was placed in an ice–water bath and exposed for about 30 min to a light condensed from a tungsten lamp (1 kW) through a lens. The red semiquinone of the enzyme thus prepared was then mixed with various reactants placed in the side chamber. The measurements were made immediately after the mixing, since disproportionation occurs slowly as reported by Müller *et al.*[11]. The absorption spectra were recorded with a Beckman DK-2A spectrophotometer. The CD spectra were measured by use of a JASCO-ORD/UV-5 spectropolarimeter with a CD attachment. ESR measurements were made with a JES-3B X-band ESR spectrometer.

RESULTS

Complex formation between the red semiquinone of D-*amino acid oxidase and benzoate or its derivative*

It was found that the formation of the blue species upon anaerobic addition of benzoate to the red semiquinone was dependent on the concentration of the added benzoate. To examine further the relation between the two, the double reciprocal plots between the concentrations of benzoate and those of the blue species were obtained. Since the difference in the absorption at 620 nm before and after the addi-



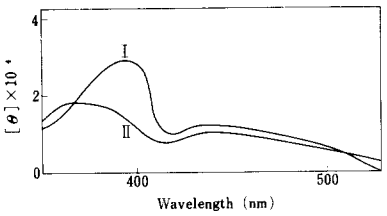
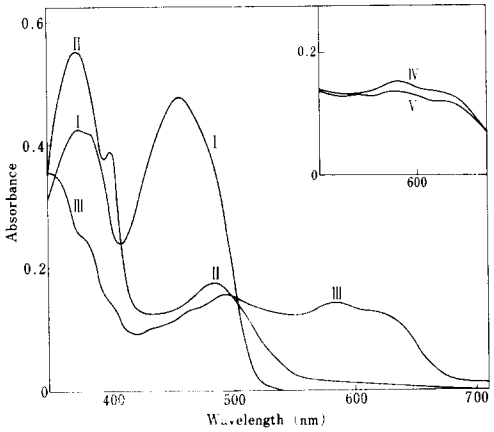


Fig. 1. Double reciprocal plots between the amount of blue species formed and the concentrations of benzoate added in the absence and presence of D-alanine. The red semiquinone prepared by anaerobic irradiation of the oxidized enzyme ($3.88 \cdot 10^{-5}$ M with respect to the bound FAD) in the presence of 0.02 M EDTA at pH 8.3 and 2 °C was mixed with various concentrations of sodium benzoate in the absence (I) and presence of $2.0 \cdot 10^{-2}$ M D-alanine (II) and the resulting increase in absorbance at 620 nm was measured.

Fig. 2. Change of CD spectrum of the red semiquinone of D-amino acid oxidase upon addition of excess benzoate. Curve I: CD spectrum of the red semiquinone prepared by anaerobic irradiation of the oxidized enzyme ($7.78 \cdot 10^{-5}$ M with respect to the bound FAD) in the presence of 0.02 M EDTA at pH 8.3 and 2 °C; Curve II: I was mixed with $1.0 \cdot 10^{-1}$ M sodium benzoate anaerobically.



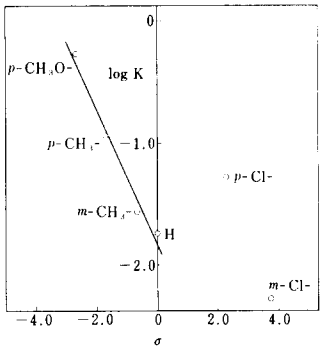


Fig. 3. Effect of pH on the conversion of the red semiquinone to the blue species of D-amino acid oxidase upon addition of excess benzoate. The oxidized enzyme ($4.20 \cdot 10^{-5}$ M with respect to the bound FAD) (I) was irradiated anaerobically in the presence of 0.02 M EDTA at pH 8.3 and 2 °C (II), and then mixed with triturated sodium benzoate in the side chamber ($1.0 \cdot 10^{-1}$ M, final concn) (III). The same procedure was performed with the enzyme ($4.50 \cdot 10^{-5}$ M with respect to the bound FAD) at pH 7.0 (IV) and pH 6.0 (V). Sodium benzoate was previously dissolved in the buffer of corresponding pH value and dried in the side chamber (see text).

Fig. 4. Hammett's plot for the complex formation between benzoate derivatives and the red semiquinone of D-amino acid oxidase. The logarithms of the dissociation constants of the complexes are plotted against σ values of benzoate derivatives. The dissociation constant was determined from the double reciprocal plots between the values of $\Delta A_{620\ nm}$ and the concentrations of benzoate derivative. Enzyme concentration was $3.92 \cdot 10^{-5}$ M with respect to the bound FAD.

tion of benzoate to the red semiquinone was a measure of the concentration of the blue species (see Fig. 3, Curves II and III), double reciprocal plots[12] were obtained between the values of $\Delta A_{620\ nm}$ and benzoate concentrations as shown by Curve I in Fig. 1. The straight line indicated complex formation between the red semiquinone

TABLE I

FORMATION OF THE BLUE SPECIES UPON ADDITION OF EXCESS BENZOATE, ITS DERIVATIVES AND OTHER CARBOXYLATES TO THE RED SEMIQUINONE OF D-AMINO ACID OXIDASE

Benzoate, its derivatives or other carboxylates ($1.0 \cdot 10^{-1}$ M, final concn) were mixed with the red semiquinone prepared by anaerobic irradiation of D-amino acid oxidase in the presence of 0.02 M EDTA at pH 8.3 and 2 °C. The resulting increase in absorbance at 620 nm was measured. Enzyme concentration was $3.88 \cdot 10^{-5}$ M with respect to the bound FAD. N.S., no significant change was observed.

	K (μM)*	$\Delta A_{620\ nm}$
Benzoate	3	0.114
o-Chloro-	800	0.013
m-Chloro-	0.36	0.129
p-Chloro-	1.6	0.082
m-Iodo-	2.7	0.048
p-Iodo-	100	N.S.
o-Methyl-	3000	N.S.
m-Methyl-	0.43	0.124
p-Methyl-	2.6	0.079
o-Amino-	20	N.S.
m-Amino-	32	0.060
p-Amino-	340	0.006
o-Nitro-		N.S.
m-Nitro-		N.S.
p-Nitro-	8	N.S.
p-Methoxy-	43	0.013
p-Hydroxy-	190	0.004
Cinnamate	55	0.033
Phenylacetate		N.S.
Crotonate	70	0.024
Butyrate	2100**	N.S.
Sorbate	180	N.S.

* Dissociation constant of the complex of substance examined with the oxidized enzyme. From the data obtained by Massey and Ganther[13], except for the value of butyrate.

** From the data obtained by Yagi *et al.*[14].

and benzoate. From the figure, the dissociation constant of the complex was calculated to be $2 \cdot 10^{-2}$ M. The complex showed paramagnetic properties as reported in previous paper[3].

Fig. 2, Curve I shows the CD spectrum of the red semiquinone. The addition of excess benzoate under anaerobic conditions resulted in a characteristic change in CD spectrum as shown by Curve II, corresponding to the change in absorption spectrum.

The effect of pH on the formation of the red semiquinone was examined. The results indicated that anaerobic irradiation of this enzyme in acidic or alkaline medium resulted in a similar absorption spectrum, and did not result in the formation of the blue semiquinone, essentially in accord with the data of Massey and Palmer[7]. An excess of benzoate was added to the solution of the red semiquinone prepared at different pH values. The changes in spectra are shown in Fig. 3, indicating the formation of the blue species at all pH values examined.

To obtain further information on the reaction of benzoate with the red semiquinone of this enzyme, the formation of the blue species with various derivatives of benzoate was examined. Table I shows the increase in $A_{620\ nm}$ upon addition of ben-

zoate derivatives ($1.0 \cdot 10^{-1}$ M, final concn). A tendency for the blue species to be formed upon mixing with *m*- or *p*-substituted benzoate, but not with *o*-substituted one, was observed. This might be explained by the so-called ortho effect.

The relation between the dissociation constant of the complex (blue semiquinone) formed with *m*- or *p*-substituted benzoate derivative and the empirical value, Hammett's σ (ref. 15) was investigated. The dissociation constant of the complex was calculated from the double reciprocal plots[12] between the values of $\Delta A_{620\ nm}$ and the concentrations of benzoate derivative. Fig. 4 shows the plots of the logarithms of the dissociation constants of the complexes against the σ values. In a certain range of σ values (−0.4–0), the points lie almost on a straight line ($r = -0.99$). From the slope of the line, the ϱ value was calculated to be −5.7. From these results, it can be concluded that the formation of the blue semiquinone complex increases with the electron-attracting nature of the carboxyl group of the substituents.

Complex formation of the red semiquinone of D-*amino acid oxidase with other substances*

Formation of the complex between the red semiquinone of D-amino acid oxidase and benzoate led us to examine other substances which combine with the oxidized form of this enzyme, because benzoate is one of the substances which form complexes with the oxidized form of this enzyme. Fatty acids also form complexes with the oxidized form of D-amino acid oxidase[14]. As shown in Table I, it is clear that a substance which forms the blue semiquinone complex has a small dissociation constant for its complex with the oxidized enzyme.

Our previous result[3], that D-alanine lowered the formation of blue color upon addition of benzoate, suggests the formation of a complex between the red semiquinone and various α-amino acids. This was examined in the present study. The absorption peak at 400 nm of the red semiquinone disappeared, accompanied by some hyperchromism around the 375 nm peak, when mixed with D-α-aminobutyrate, DL-α-aminocaproate or L-alanine as already observed with D-alanine[3]. This indicated that α-amino acids interact with the red semiquinone to form complexes. To further support the view that these amino acids form complexes with the red semiquinone, the effect of D-alanine on the formation of the blue complex with benzoate was analyzed. As shown by Curve II in Fig. 1, D-alanine competed with benzoate in the formation of the blue semiquinone complex. The result shows that the binding site for benzoate on the red semiquinone involved in forming the blue species is identical or overlapping with that for D-alanine in the formation of the red semiquinone–D-alanine complex. The dissociation constant of the complex of D-alanine with the red semiquinone was calculated to be $2.8 \cdot 10^{-3}$ M. Similar results were also obtained with L-alanine.

DISCUSSION

The present data clearly demonstrate that the red semiquinone of D-amino acid oxidase is changed into the blue species upon mixing with excess benzoate or its derivatives. Comparing the absorption spectra of these two species with those of anionic and neutral radicals[16], it can be concluded that the flavin moieties of these two species are in the form of an anionic and a neutral flavin radical, respectively. Even though glucose oxidase was converted to either blue or red semiquinone upon anaerobic irradiation depending on solution pH, such a phenomenon was not observed

in the case of D-amino acid oxidase, in accord with the data of Massey and Palmer[7]. Moreover, the present data indicate that preparations of the red semiquinone of the enzyme obtained at various pH values all changed into the blue species upon addition of benzoate. It was also shown that this change is caused by the binding of benzoate or its derivatives to the red semiquinone. Therefore, the change of the semiquinoid enzyme from red to blue, *viz.* the protonation of the anionic radical upon complex formation with benzoate cannot be explained by assuming that the flavin moiety is exposed to medium water and protonated upon lowering the pH of the solution but can be explained by supposing that the change in the environment surrounding the flavin chromophore due to protein conformational change is induced by complex formation with benzoate, as already predicted[17].

Another important point that should be emphasized in the present results is the fact that the electron-attracting nature of the carboxyl group of benzoate derivatives determines the degree of formation of the blue species. This result indicates the participation of the COO^- group in the conversion of the anionic radical to the neutral radical in the present study. However, it does not necessarily show that the COO^- group of the reactant reacts directly with the anionic radical. Conversely, it seems reasonable to consider that the COO^- group of the compounds examined combines with a positively charged group on the protein, since substrates such as D-alanine, in addition to inhibitors having a COO^- group such as L-amino acids, all combine with the red semiquinone of the enzyme. However, carboxylates induce the conversion of the semiquinoid enzyme from red to blue, *viz.* protonation of the anionic radical, while amino acids do not. The view that participation of the COO^- group could be involved in complex formation between the carboxylates examined and the red semiquinone of the enzyme can be supported by the fact that the dissociation constant of the complex between the carboxylates examined and the oxidized enzyme seems to be inversely related to the degree of formation of the blue species. In this connection, it should be recalled that the electron-attracting nature of the carboxyl group of benzoate derivatives determines the degree of inhibitory action on this enzyme[18], in other words, the degree of complex formation with the enzyme.

All these results indicate that the conversion of the red semiquinone to the blue semiquinone of D-amino acid oxidase is induced by formation of a complex between the red semiquinone and a carboxylate possessing a strong electron-attracting nature.

Although the detailed mechanism of protonation of the anionic radical due to a change in the environment surrounding the flavin chromophore is not known, one possibility could be the interaction between the protein residue and the flavin radical as predicted by Müller *et al.*[16]. Since the highest spin density is located at N-5 of the flavin radical[16], N-5 should be the interacting site of the flavin moiety and the proton-withdrawing and proton-donating residues might be responsible for the stabilization of the anionic and neutral radicals of flavin, respectively. If these suppositions are valid, it is considered that complex formation between carboxylate and the red semiquinone of the enzyme may induce dislocation of some proton-withdrawing residue, previously interacting with N-5 of the anionic radical, and approach of some proton-donating residue to the same N-5 position. The fact that amino acids combine with the red semiquinone of the enzyme without protonation of the anionic radical may be explained by the interaction of their amino group with N-5 of the anionic radical. As another explanation for the protonation of the anionic radical upon formation of

the complex, it might be supposed that some residue interacting with N-5 of the anionic radical changes from proton-withdrawing to proton-donating due to the complex formation. Although Müller *et al.*[16] proposed for glucose oxidase that the imidazole group of some histidyl residue may interact with N-5 of the flavin radical and that regulation of the charge on the radical without removal of the stabilizing group from N-5 may be possible due to a change in solution pH, such a pH dependence is not found for D-amino acid oxidase as discussed before. If the regulation of the charge on the radical by an imidazole group has to be assumed in the case of D-amino acid oxidase, this must be ascribed to the protein conformational change brought about by complex formation with carboxylates.

In any case, direct interaction of the COO^- group of the carboxylate with N-5 of the anionic radical may be ruled out, in so far as the interaction of a proton-donating residue is required for the conversion of the red semiquinone to the blue semiquinone of D-amino acid oxidase.

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